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STAIN TECHNOLOGY

OCTOBER, 1928

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STAIN TECHNOLOGY

VOLUME III OCTOBER, 1928 NUMBER 4

PROGRESS IN THE STANDARDIZATION OF STAINS

FIVE YEARS OF STAIN STANDARDIZATION

The first certified stain, methylene blue, was put on the market in the summer of 1923. Five years have passed since then and it is interesting to take account of stock, and to see the progress that has been made.

During the first two years of this period the chairman had at his disposal no assistance other than clerical help and at the beginning this was available for only half time. With such meager assistance as this, the work had to begin slowly, as no one in the chairman's laboratory had time to do much in the way of testing stains that were submitted for certification, nor for that matter, experience in a sufficient variety of histological work to form an authoritative opinion on any of the dyes. All samples received had to be sent out to other laboratories for testing and all the work done had to be entirely voluntary. Under such circumstances, it was plain that the Commission could not test any sample of any stain that some company might choose to send in without previous notification. Instead it was necessary to adopt a policy of putting the stains one by one on the certification basis and inviting the stain companies to submit samples of each of these dyes in turn at definitely specified dates. Little by little the number of stains on this basis was increased as the Commission members obtained greater familiarity with the dyes, and as the facilities for testing them increased, until now all the commonly used stains are on the certification basis and the stain companies are free to submit a sample of any of these dyes at any time.

It is interesting to notice the increase in the number of stains on the certification basis during this period. This increase is plainly shown in Table 1. It will be seen that during 1923 only two different stains were certified, namely, methylene blue and safranin. During 1924 eight more were added to the list. By this time, therefore, ten of the most commonly used stains were on the market bearing the Commission's certification label, and the latter was already becoming well known among biologists in this country. It was felt, however, that the progress of the work was altogether too slow. At the rate of increase observed that year, it would be a long time before all

the common stains could be certified, and it was realized that as soon as the companies began submitting new batches of the first dyes certified, there would be more work involved than the Commission could handle. Furthermore, in the case of these first ten dyes, several months were found necessary to secure adequate judgment as to the reliability of a sample. Before that time was over, the stain company might have sold out the entire batch, and all the work done on it would, therefore, be useless. For this reason it was felt that something must be done to speed up the work, and to increase the amount of it that could be handled.

TABLE 1. LIST OF STAINS ON CERTIFICATION BASIS PREVIOUS TO JAN. 1, 1928.

First certified in 1923	First Certified in 1924	First Certified in 1925	First Certified in 1926	First Certified in 1927
Methylene blue	Crystal violet	Anilin blue, W.S.	Brilliant green	Ethyl eosin
Safranin O	Eosin Y	Bismarck brown Y	Eosin B	Martius yellow
	Fuchsin, acid	Bril. cresyl blue	Jenner's stain	Methyl violet
	Fuchsin, basic	Carmin	Malachite green	Methylene violet
	Gentian violet	Congo red	Nile blue A	
	Hematoxylin	Cresyl violet	Orange II	
	Orange G	Indigo carmin	Nigrosin	
	Pyronin	Janus green B	Phloxine	
		Light green SF yellowish		
		Methyl green		
		Methyl orange		
		Methylene azure		
		Neutral red		
		Sudan III		
		Sudan IV		
		Tetrachrome stain		
		Thionin		
		Toluidine blue		
		Wright's stain		

This became possible in 1925, thanks to the ever increasing liberality of the Chemical Foundation and to the willingness of the New York Agricultural Experiment Station to put additional space at the disposal of the Stain Commission. In this year a histologist was added to the Commission staff, who has remained with us ever since, and has in the meantime secured a very wide experience in the use of dyes for a large variety of biological purposes. As a result of this increased assistance, 18 more dyes were placed on the certification

basis during 1925, thus increasing the number of certified stains on the market up to 29 by the end of the year. By the end of 1926 this number had increased to 37 and by the end of 1927 up to 41. It will be seen from Table 1 that the complete list of stains certified up to the end of that year covers practically all that a biologist ordinarily requires. Since the beginning of 1928 one more stain, rose bengal, has been added to the list.

Another way in which progress has been made, but which is not shown on the table, is the speed with which the work can be dispatched. While four or five months were necessary to test the first samples received from 1923 to 1924, a report is now ordinarily possible on a dye within three or four weeks after its receipt by the Commission. This greater speed is due to the fact that much more of the work can be done in the Commission laboratory, and it is less frequently necessary to send samples out to collaborators elsewhere. As a result, our reports reach the stain companies before they have to begin marketing the batch which is being tested. This makes it possible even for the smaller companies to submit a greater variety of dyes for certification, and it accordingly increases greatly the number of certified dyes on the market.

This increase is reflected in a general way by the number of certification labels which the Commission has furnished the stain companies, altho without certain explanations the figures are slightly misleading. It must be explained that in 1925 to 1926 one of the stain companies was stocking up on certified stains for several years in advance, and purchased, therefore, a larger number of labels than it has since then. The number of labels furnished the companies up to July 1st of this year are as follows:

1923—	2,000
1924—	3,850
1925—	13,234
1926—	19,060
1927—	10,386
(first six months) 1928—	6,000
Total	54,530

The decrease in 1927 is for the reason explained above. The general increase thruout the period is evident, however; and the company that purchased so many labels in 1925–1926 assures us that their stocks are getting low and that they will have to repeat the process of stocking up this year or next, thus making an increase in the number of labels sold quite probable in 1929.

Not all of the samples submitted for certification have been satisfactory, and thruout the five years a certain proportion of the samples has been rejected. The total number of samples submitted during this period has been 250 and of these 36 have been rejected. A study of the number of samples rejected each year, as shown in Table 2, indicates a distinct improvement in the stain supply during the period. It will be seen that the number of samples rejected has decreased almost steadily from 45% of the total number submitted in 1923 to 3% of the number submitted during the first half of the present year. When in addition to this fact, it is realized that there has been in the same period a great decrease in the number of different kinds of dyes not on the certification basis, it becomes still more plain that there must have been a great improvement in the supply available on the American market.

Questions are frequently asked as to just what certification of a stain means. This question is best answered by giving the procedure which is followed in handling the samples submitted for the purpose.

TABLE 2. IMPROVEMENT IN STAIN SUPPLY SHOWN BY DECREASE IN SAMPLES REFUSED CERTIFICATION

	1923	1924	1925	1926	1927	1928 1st 6 mos.
Number of samples tested	20	46	39	45	57	33
Number of samples rejected	9	14	5	3	7	2
Percent rejected	45%	30%	13%	7%	12%	3%

The sample of any batch of stain submitted is sent to the Commission by the manufacturer, packed in two bottles of 10 grams each and five bottles of 1 gram. These individual bottles are handled as follows: One of the 10-gram samples is placed on file for future reference in case it becomes desirable at any time to compare this batch of stain with a later lot. The other 10-gram sample is sent to the Color Laboratory of the Department of Agriculture at Washington, whose chemists have been coöperating with the Commission thruout the work. At this laboratory the percent dye content of the sample is determined, and a spectrophotometric analysis is made to determine whether the dye is optically true to type. One of the 1-gram samples is tested in the Commission laboratory in such procedures as its members are familiar with. The other 1-gram samples are used for sending out to collaborators for testing in procedures of which the staff of the Commission laboratory are not familiar. With many of the dyes now it is not necessary to distribute any of the

samples in this way; with others it is still important to send out two or three samples to different laboratories for testing. As soon as the reports are all in, a form is sent to the stain company informing them as to whether the sample is approved or rejected, and, if it is approved, they are then furnished with as many certification labels as they may wish to order.

This procedure, as will be seen, trusts to the honesty of the company in not using the labels furnished them for any other batch except the one for which it was issued. Theoretically, this leaves something to be desired, since dishonesty is possible. Practically, however, our experience with the integrity of all the companies submitting samples has shown us that no concern on this account is necessary and that fraudulent use of the labels furnished them is extremely unlikely. If misuse of the labels ever occurs, it is not intentional, but merely thru the carelessness of some employee, and no evidence has as yet been received that even the latter has occurred.

All told, there seems abundant reason to be satisfied with the progress of the five years just completed. The thanks for this are due to the whole-hearted cooperation of the stain manufacturers, to the generosity of the Chemical Foundation, to the personal interest shown by its president, Mr. F. P. Garvan, to the facilities furnished for the work by the New York Agricultural Experiment Station, to the coöperation of the Color Laboratory of the United States Department of Agriculture, and finally to the widespread interest and assistance given by biologists thruout the country, too numerous to mention by name.

H. J. Conn

THE HISTORY OF STAINING

COCHINEAL DYES

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Of the dyes used in early histological work, none was so highly prized as carmin. Even today biologists would be loath to part with it. It is still valuable to the histologist and embryologist as a bulk stain of great permanency, and for the preparation of *in toto* mounts. To the cytologist, also, it is invaluable as a medium for the rapid staining and examination of chromosomes in fresh material. Its permanence has always been a great advantage. To the early histologists, however, it was *the* dye, *par excellence.*

One can best realize the important part which carmin played in early histological work by the very casual way in which the name of this dye is mentioned by Hartig (1854), as translated in the preceding paper of this series.[1] Hartig first mentions carmin in this paper as the "dye solution," and does not call it by name until a few pages later. There were other natural dyes known in those days, primarily madder, indigo, and logwood extract; but in comparison with carmin they gave such unsatisfactory results that they were not highly regarded. It seems strange to realize what little attention was paid to logwood, which is now of greater value in histology than cochineal. It must be remembered, however, that before microscopists began using dyes, carmin had already been prepared commercially from cochineal; while an equally satisfactory product from logwood was not yet known.

As discussed in the preceding paper, cochineal was used in microscopic work as early as 1770 by Hill, later by Ehrenberg[2] (1838) in studying protozoa, then by Göppert and Cohn (1849) in studying the cell contents of *Nitella flexilis* and by Corti (1851) in examining the epithelial lining of the cochlea. It first became an important tool in microscopical work in the hands of Hartig, who made an extensive investigation of the ability of different parts of the plant cells to take up carmin; following this, the work of Gerlach showed more practical methods of employing it, while that of Maschke proposed a very plausible theory of its action. All these contribu-

[1]H. J. Conn. The Pioneers in Staining. Stain Techn., 3, 1–11. See p. 2. An exact reference to Hartig's paper may be found in the list of references at the end of the article cited.

[2]References to all papers earlier than 1860 to be found at end of article cited in footnote 1. Later references given at the end of this paper (p. 121).

tions have been discussed in the preceding paper; but the work of Gerlach and his followers must be taken up in more detail.

Nature of Cochineal and Carmin

Before discussing the history of the use of this dye, it is very interesting to consider its origin and the method of manufacture. Carmin, as is well known, is derived from cochineal, and the latter is a product obtained by drying and grinding the bodies of a certain insect. The cochineal insect, *Coccus cacti*, is a native of Mexico. It was cultivated and used for dyeing in that country long before the Spanish conquest. It has subsequently been introduced to other tropical countries and is now quite widely cultivated.

The cochineal insect is very small, not over 1/12 inch long. The males are smaller than the females, and only one male is produced for about 300 females. The greater abundance of the female is important from the practical standpoint, for the male does not contain the dye for which the species is prized. The dye seems to be present in the female in the form of a purple colored sap. The insect multiplies very rapidly, and in the tropics where it is raised, 4 or 5 broods a year may be obtained. Just before egg-laying, the females are almost spherical in shape, and it is then that they are harvested. They are scraped from the plants where they live, killed and dried. There is considerable difference in the quality of the cochineal obtained from different places, the product from the wild insects not proving as good as that obtained from the cultivated forms. Pulverized insects, sold as cochineal, are often highly adulterated.

Carmin is derived from cochineal by boiling with some salt that causes a precipitate of a partially purified product. Alum is generally used for this purpose, but methods for manufacture vary. As a result of the various methods of preparing carmin, this dye can vary more than cochineal itself. It was early realized that not all grades of carmin were equally good for microscopic work. Mayer (1892) has given several tests to determine the purity of the products.

The dye in carmin is carminic acid, a compound whose exact composition is not yet known. It is possible to obtain a fairly pure carminic acid from carmin, and this is now available to biologists from their supply houses. Carminic acid is preferred by many on account of its greater purity and uniformity, and is called for in some of the most valuable formulae. It is generally considered, however, that a good grade of carmin gives as satisfactory staining results as does carminic acid.

Gerlach and His Followers

Gerlach's historic papers on the use of carmin were published in 1858. In one of them he states that as much as four years previously he had injected the blood vessels of animals with ammoniacal carmin which diffused into the walls of the vessels; he had observed that the nuclei stained more deeply than the rest of the cells and the intercellular substance. Encouraged by that, he tried staining sections of the central nervous system with concentrated carmin solution, but did not get satisfactory results. By accident he left a section of cerebellum standing over night in a dilute solution, and upon subsequent examination he noticed its striking differentiation. He also made attempts, published in another paper, to stain living tissue of animals. He never succeeded. He found that dead tissue withdraws practically all the color from a dilute carmin solution, which enters mostly into the nuclei and nuclear granules, less into the body of the cell and least into the intercellular substance. The dye cannot be washed out. He therefore concluded that there seemed to be a specific affinity between the dye and the tissue elements, for which no physical explanation could be given.

In all this, Gerlach was largely verifying for animal tissue what Hartig had already found out in regard to plants, as discussed in the first paper of this series. We also saw, in this same paper, how Maschke one year later, without having seen Gerlach's work, discussed the theory of staining with carmin and prophesied a very important future for the practice of staining.

Carmin is not soluble in water. To use it as a stain, therefore, one must obtain some derivative of it which is soluble. The two most commonly used compounds are ammoniacal carmin and acetocarmin. Gerlach, as we have seen, employed the former. A modified formula for ammoniacal carmin, with oxalic acid, was published by Thiersch, who also employed a boric carmin. The formulae of Thiersch appeared in the literature in an editorial note in Schultz' Arch. f. Mikro. Anat. (Schultz, 1865). Two of these formulae seem worth giving here, as they are among the earliest useful carmin formulae.

The oxalic formula is:

1 pt. carmin
1 pt. ammonia
3 pts. distilled water

Mix 1 volume with 8 volumes 1:22 aqueous oxalic acid, then 12 vols. absolute alcohol. Filter. This was used for sections.

The formula with borax is:

1 pt. borax
56 pts. distilled water
1 pt. carmin

Mix 1 volume with 2 volumes absolute alcohol. Filter. This was used for bone and cartilage hardened in chromic acid. May be decolorized, if overstained, in a solution of borax or oxalic acid in spirits of wine.

About this same time an interesting formula for ammoniacal carmin was given by Beale. According to Gierke this formula was given by Beale in some of the early editions of his book altho the 5th edition (1880) is the earliest one to which Gierke has a definite reference. The formula is as follows:

10 gram. Carmin
½ drachm. Liq. Ammon. Caust.
2 oz. glycerin
2 oz. Aq. dist.
½ oz. alcohol

The carmin is shaken in a test tube with the ammonia and boiled a few minutes, and a few hours later the water, glycerin and alcohol are added. The solution is filtered and filtrate may be preserved for several months.

Schweiger-Seidel and Dogiel (1866) employed an acetic carmin prepared as follows: Ordinary ammoniacal carmin is added to an excess of acetic acid until a wine-red liquid results, and is then filtered. Preparations are diffusely stained with this solution and decolorized with glycerin containing 0.5% hydrochloric acid.

At about the same time Schwartz (1867) employed carmin with picric acid as a double stain. The two dyes were applied separately in his procedure. Sections of fixed tissue were placed for five minutes in a very dilute acetic acid then transferred to the following carmin solution:

0.203 gr. carmin
60 drops ammonia
250 c.c. water

After standing in this fluid 24 hours they were washed in distilled water and placed for 2 hours in picric acid (0.066 gr. to 200 c.c. water). Schwartz' article is accompanied by some very striking colored illustrations to show the staining effects secured on different types of tissue. The writer also used some green dye for contrast to carmin in place of picric acid, but does not seem to give any information by which this dye may be identified.

Ranvier (1868) the following year is apparently the first to employ carmin and picric acid in a single staining fluid. He does not give exact directions for its preparation, but makes a statement about it which translated is as follows:

"When one adds to a concentrated solution of picric acid a solution of carmin in ammonia until the liquid assumes the shade of the juice of red currants, one obtains a staining fluid valuable for the study of delicate details."

According to Gierke (1884, p. 86) Betz (1872) employed a carmin solution ripened by standing in sunlight until a dirty red, flocculent precipitate was formed. This solution was then filtered and the filtrate used.

In 1872 Woodward proposed a modification of Thiersch's borax-carmin. The original publication of Woodward's has not been seen, nor was it apparently available to Gierke, but the matter is given in an editorial note (Lawson, 1872) in the *Monthly Microscopic Journal*. Woodward showed that the filtrate employed by Thiersch is a weak stain, altho valuable. He found that the bulk of this dye remained in the precipitate, which stains energetically if redissolved in distilled water. He showed that the same effects may be secured by mixing carmin and borax in water without precipitating, but that this mixture is not stable while the solution of precipitated borax-carmin is stable.

Lieberkühn (1874) injected dyes into the blood vessels of dogs and the lymph sacs of frogs to observe what portions of their bodies became colored. In this way he employed both alizarin and carmin. Translations of two rather interesting passages concerning this latter dye follow:

"It now seemed appropriate to repeat the well-known experiments of Gerlach with carmin; I can only reaffirm that as declared by this investigator, frogs do not take up any color when placed in an aqueous solution of this dye. But if, with a Pravaz syringe, one fills the lymph sac of the back or of the abdomen, then the whole frog becomes red, even including his back, if he is weakly pigmented. Six days after the injection the color is still pronounced. . . .

Prof. A. W. Hoffman kindly furnished me with a considerable quantity of arsenic-free anilin. Frogs that were injected in the abdomen with an aqueous solution from a Pravaz syringe decolorize in a few days; only in the fatty tissue is a little of the dye left."

Hoyer (1877) found he could obtain better results with an alcoholic-carmin solution than with the aqueous ammoniacal carmin, when using the solution for injection. In this way, he could obtain, instead of a diffuse coloring of the whole injected part, a selective staining of the walls of the blood vessels. He obtained an especially finely stained section of tissue injected with carmin and berlin blue in alcohol (with or without acetic acid). For injection he employed a carmin solution containing only a slight excess of ammonia to which was added about 20–25% of alcohol (one part carmin to 80

parts solution and 20 parts alcohol). He obtained an intensive stain by dissolving carmin in alcohol with heat, precipitating with sugar of lead, suspending the precipitate in alcohol and dissolving by adding to it drop by drop alcohol, acidified with sulphuric acid.

Mayer (1878) proposed a simple tincture of cochineal. He employed one gram of powdered cochineal in 8–10 c.c. and 70% alcohol. This solution was filtered after several days and the filtrate used for staining. Mayer refers to this as a splendid nuclear stain. It should be remarked that we owe to Mayer most of our exact knowledge about cochineal products, and that his formulae are still highly prized.

The same year Obersteiner (1878) proposed the following method for securing the rapid staining of sections of the central nervous system: The sections are placed in a carmin solution in watch-glasses, which rest on a net suspended over a boiling water bath. The sections are thus completely stained after 2 to 5 minutes. They are then washed twice in distilled water and prepared for mounting. The writer states that the whole procedure requires not more than 10 minutes; and Gierke, reviewing the matter, agrees that the procedure is very good when speed is desired. Gierke adds, however, that the method is not to be employed unless one wishes quick results.

Grenacher (1879) discussed the unreliability of commercial carmin. This is interesting, as it shows that the microscopist of those days realized that there was a problem in securing dependable dyes, just as we do today. In one respect this problem was greater then than at present, because no dyes were prepared especially for biologists and the methods of manufacture then were very crude; on the other hand, microscopists did not require so much in the way of performance from the dyes as they do with the greatly diversified technic of today. In order to obtain a more reliable product, Grenacher boiled a solution of alum or ammonia-alum (1–5% or stronger) with 0.5–1% of powdered carmin for about 10 to 20 minutes; this solution was filtered after cooling. The solution has a very rapid action, staining the nuclei alone a distinct purple; even on long standing no overstaining takes place.

Grenacher also proposed the following modification of the Schweiger-Seidel method:

1–2% of borax is boiled in water with about 0.5–0.75% of carmin and a dark purple solution results. Dilute acetic acid is added to this drop by drop with constant shaking until the color changes to a deep red, the same shade as the usual ammoniacal carmin solution. It is filtered after 24 hours.

This solution has a diffuse staining action. In order to limit the staining to the nuclei, the writer washed the sections in watch-glasses containing 50–70% alcohol to which a drop of hydrochloric acid had been added. A simply prepared solution was also proposed by Grenacher:

Alcohol (60–80%) is acidified with hydrochloric acid (3–4 drops to 50 c.c.), and a small quantity of carmin (as much as could be held on the point of a knife blade) is boiled in it for about 10 minutes. The solution is filtered after cooling. The writer gives directions for assuring the proper reaction of this solution by the addition of hydrochloric acid or ammonia.

It is claimed by Gierke that neither of these last two solutions were of practical value. They are given here partly to illustrate the variety of ways in which the early users of stains tried to secure the most satisfactory results with carmin, and partly because a modification of the last formula, proposed by Mayer, proves very useful. Mayer boils 4 g. carmin in 15 c.c. water with 30 drops of hydrochloric acid and adds 95 c.c. of 85% alcohol. Filters while hot. Neutralizes by adding ammonia until a permanent precipitate forms; cools and filters. This is an extremely useful stain for embryological work either for staining in bulk with subsequent sectioning or for *in toto* mounts.

Schneider (1880) prepared an aceto-carmin in the following way: Carmin is added to boiling 45% acetic acid until no more dissolves, and is then filtered. The writer states that one may dilute this to 1% and apply for a considerable period of time, or a drop of the concentrated solution may be placed directly under the cover glass. He employed this solution in studying cell division of germinating eggs. This solution has very extensive use today among cytologists for the rapid examination of chromosomes in fresh tissue. The addition of a minute quantity of iron chloride (Belling, 1921) gives an almost black tinge to the chromosomes.

Czokor (1880) complained of the quality of the commercial carmin and laid even more stress upon its unreliability than Grenacher had the year before. He suggested that a more reliable product might be made by the microscopist himself. His method was as follows:

In a mortar 7 grams of cochineal and 7 grams of alum are ground to a powder; 700 grams of distilled water are added and the solution boiled down to about 400 grams. After cooling a trace of phenol is added (so as to preserve it) and the solution is filtered. The filtrate is a violet solution and may be kept for half a year, when it must be refiltered and another trace of phenol added.

This solution, as shown by Czokor and reaffirmed by Gierke, is a splendid substitute for carmin and gives the nuclei about the same

color as does hematoxylin. The only drawback is that the solution does not keep very well, a precipitate forming very readily in warm weather.

Hoyer (1882) insisted that a pressing need was a preparation of ammonium carminate in a dry form that would always be ready for use at any time in any desired concentration.

To prepare this he dissolved 1 gram carmin in 1–2 c.c. strong ammonia and 6–8 c.c. distilled water, heating until the excess ammonia is driven off, cooling and filtering. The filtrate contains the ammonium carminate and if desired may be preserved by adding to it 1% or more of chloral hydrate, or the dye may be precipitated out by adding to this solution 4–6 times its volume of alcohol. The copious precipitate may be separated out by filtration and preserved indefinitely as a dry powder or it may be converted into a paste with the addition of alcohol, glycerin and chloral hydrate.

Gierke states that about this same time, probably in 1882, the apothecary Maschke put on sale various carmin preparations, among which was a sodium carminate in dry form. Gierke says that this preparation can be used much the same as Hoyer's ammonium carminate and it is in some respects more convenient to employ.

Later Developments

As Gierke's comprehensive summary of the history of staining was written in 1883, this date forms a convenient point for separating the early period from the later period. By this time the pioneer work had been finished, and further developments consisted largely in the refinement of procedures already employed. No desirable purpose would be accomplished by reviewing all the carmin formulae proposed after this date. There are, however, a few outstanding procedures that should be referred to.

One of the important developments was its wide application to cytological studies. It was thus employed earlier than 1883. Flemming, who is considered the father of modern cytological technic, used this dye in his studies on the egg of Anodonta (1874). Hertwig (1876) also employed carmin in his classical research on the fertilization of the echinoderm ovum, as did Van Beneden (1883) in his most remarkable study of the maturation, fertilization, and cleavage of Ascaris. Boveri (1887, 1890) in his first three important cell studies, used carmin almost exclusively and found that the acetic carmin of Schneider (see p. 116) was very valuable.

Lee in the first edition of his Vade-Mecum (1885) makes the following very interesting statement:

"Carmin, which is probably the most valuable and certainly is the most widely employed of histological coloring agents, was first proposed as an aid in examining animal tissues by Gerlach in 1858,

since which time, notwithstanding the discovery of numerous other substances that have proved most useful in many kinds of research, it has held its place with a firmness that shows few signs of yielding as a staining agent *par excellence*."

One of the most useful formulae was proposed almost contemporaneously with Gierke's paper and is therefore not referred to by this author. This formula in question is the lithia carmin of Orth (1883). To a cold saturated solution of lithium carbonate Orth states that one may add "almost any desired quantity" of carmin. Tissues or sections are differentiated in acid alcohol after staining. The solution does not need to be filtered, and Orth states that it does not spoil, altho modern statements of the formula generally call for the addition of thymol as a preservative. Orth states that in his earlier work he employed 5% of carmin in this formula, but subsequently changed to 2.5%. Tissues or sections are differentiated in acid alcohol after staining.

An extremely interesting and valuable paper on carmin was that of Mayer's (1892) in which several of his most important formulae are proposed. In this paper he first discusses commercial carmin and shows that it is essentially an impure aluminium compound of carminic acid. Like Grenacher and Czokor, Mayer complains that the products on the market varied and did not give reliable results.

Secondly, Mayer discusses carminic acid. He describes it in a general way and states that chemists are not familiar with its salts. He shows that one may obtain its calcium or aluminium salts by adding the chloride of one of these bases to carminic acid or better to its ammonium salt. Mayer proposed at this time several carminic acid solutions which are still regarded as very useful. The best known of these is the one which he named "Carmalum" the composition of which is as follows:

Carminic acid —1 gram
Alum —10 "
Distilled water—200 c.c.

Dissolve by heating, pour off clear, or filter. This solution remains clear if an antiseptic is added (for example a few crystals of thymol or 0.10% salicylic acid).

Mayer also states that a deep red may be obtained by adding calcium chloride to carmin. A troublesome precipitate of calcium results, however, which may be avoided by using aluminium chloride instead of alum. He also suggests two other solutions, of which the first is as follows:

Carminic acid—1 part
$Al\ Cl_3$ —3 parts
Water —200 parts

An antiseptic should be added, as in the case of carmalum.

The other formula is the solution which he named "Paracarmin" its composition is:

Carminic acid	—1 gram
$AlCl_3$	—0.5 gram
$CaCl_2$	—4 grams
70% alcohol	—100 c.c.

Dissolve cold or warm and filter after settling.

This solution was preferred in Mayer's laboratory for some purposes to carmalum.

Mayer lastly takes up cochineal, discussing the composition of this impure product and referring to certain formulae proposed by other writers. On account of the low price of cochineal, Mayer regarded it as desirable to find, if possible, a satisfactory formula by which it can be employed. He suggests that the following will be found to give results comparable with those obtained from paracarmin:

Cochineal	—5 grams
$CaCl_2$	—5 "
$AlCl_3$	—0.5 "
Nitric Acid (1.20 sp. gr.)	—8 drops
Alcohol (50%)	—100 c.c.

In employing this solution, one must remember that cochineal varies in its composition and that this staining fluid is therefore not as uniform or reliable as paracarmin.

This paper is of value not only on account of the formulae proposed in it, but because of its historical review of the subject and the classification given of the various carmin and carminic acid formulae already in use.

Rawitz (1899) describes various carminic acid preparations that are frequently employed. Outstanding among them is the one he calls "muci-carminic acid" and employed as a stain for mucin. Its formula is:

Carminic acid	—0.5 g.
$AlCl_3$	—1 g.
50% alcohol	—100 c.c.

These ingredients are dissolved by warming.

Another outstanding method of recent years is the iron-alum cochineal of Spuler (1901). In this method the writer stains tissue two or three days in a specially prepared aqueous-alcoholic cochineal solution; and then after washing it is placed for a day or two in a 0.75 to 1% solution of iron alum. The technic is noted for its ability to bring out fine detail.

One of the most valuable of recent uses of carmin is that of Best (1906) for staining glycogen in cells, which gives results quite unlike those usually obtained with this dye. It is as follows:

Carmin— 2.0 pts.
K_2CO_3 — 1.0 "
KCl — 5.0 "
Water —60.0 "

Boil a few minutes, but do not over-heat. After cooling add 20.0 parts ammonium hydrate. May be kept in tightly stoppered bottles a few months. Filter before use.

This stain is to be used on material fixed in Carnoy's acetic alcohol or in absolute alcohol alone embedded in celloidin, or treated with celloidin after paraffin embedding. One should first stain with hematoxylin or with hemalum, then differentiate with hydrochloric-acid-alcohol. Stain in a mixture of: 2 parts of the above carmin solution, 3 parts ammonia, and 3 parts methyl alcohol. Differentiate in a mixture of: absolute alcohol 80 parts, methyl alcohol 40 parts, distilled water 100 parts. The glycogen stains a bright red.

In summing up it becomes very evident that cochineal dyes have played an extremely important part in the history of staining. Before the discovery of anilin dyes, carmin was the only stain available that was satisfactory for histological purposes. The impetus given to its use in these early days would have assured it a place in the histological laboratory for many years after the introduction of anilin dyes, even had it proved decidedly inferior to these newer products. As a matter of fact, the cochineal dyes have proved so well adapted to certain special purposes that no artificial dye yet known promises to replace them. For bulk staining, or for staining embryos *in toto*, carmin is second to none, while the wide range of formulae available calling for cochineal, carmin or carminic acid, makes possible their adaptation to a long list of histological and cytological purposes. A certain amount of experience in their use is necessary; and many failures to secure good results have been due to the method of fixation of the tissues. Thus tissues fixed in formalin, in chromic or osmic acids stain poorly with carmin; while fixation with picric acid or bichloride of mercury allows this dye to stain splendidly. The dye is, moreover, difficult to secure in uniform quality, thanks to the fact that it is not synthetically prepared. Yet with all its disadvantages, it is one of the extremely valuable stains; and altho the first dye to be employed by the microscopist, it becomes increasingly evident that neither carmin nor the other cochineal products are yet ready to be put in the discard.

REFERENCES

Beale. 1880. How to Work with the Microscope. 5th Edition. London. (Cited from Gierke.)

Belling, John. 1921. On Counting Chromosomes in Pollen Mother Cells. Amer. Nat., **55**, 573–574.

Best, F. 1906. Über Karminfärbung des Glycogens und der Kerne. Zts. Wis. Mikr., **23**, 319–322.

Betz. 1872. Methode, feine Schnitte a. d. Centralnervensystem anzufertigen. Mitth. d. ärztl. Ver. Wien, 1872, **1**, 9. (Original not seen. Cited from Gierke.)

Boveri, Th. 1887. Zellen-Studien. Jenaische Zeitsch., **21**, 423–515; **22**, 685–882. 1890. Zellen-Studien, Id., **24**, 314–401.

Czokor, J. 1880. Die Cochenille-Carminlösung. Arch. Mikr. Anat., **18**, 412–414.

Fleming, W. 1874. Über die ersten Entwicklungserscheinungen am Ei der Teichmuschel. Arch. Mikr. Anat., **9**, 257–292.

Gierke, H. 1884, 1885. Färberei zu mikroscopischen Zwecken. Zts. Wis. Mikr., **1**, 62–100, 372–408, 497–557; **2**, 13–36, 164–221.

Grenacher, H. 1879. Einige Notizen zur Tinctionstechnik, besonders zur Kernfärbung. Arch. Mikr. Anat., **16**, 463–471.

Hertwig, Oscar. 1876. Beiträge zur Kenntnis der Bilding, Befruchtung und Teilung des tierischen Eies. Morph. Jahrb., **1**, 347–432.

Hoyer, H. 1877. Beiträge zur anatomischen und histologischen Technik. Arch. Mikr. Anat., **13**, 645–650.

1882. Beiträge zur histologischen Technik. Biol. Centbl., **2**, 17–24.

(Lawson, H.) 1872. The Best Mode of Carmine Staining in Tissues. Monthl. Micro. J., **8**, 37–38.

Lee, A. B. 1885. The Microtomist's Vademecum. (First Edition.) Blakistons, Philadelphia.

Lieberkühn. 1874. Über die Einwirkung von Alizarin auf die Gewebe des lebenden Körpers. Sitzber. d. Gesel. z. Beförd. d. Gesam. Naturwiss. z. Marburg. **1874**, 33–45.

Mayer, P. 1892. Über das Färben mit Carmin, Cochinille und Hämatein Thonerde. Mitt. a. d. Zool. Stat. Neapel, **10**, 480–504.

Obersteiner, H. 1878. Technische Notiz. Arch. Mikr. Anat., **15**, 136–138.

Orth, J. 1883. Notizen zur Färbetechnik. Berliner Klin. Wochnsch., **20**, 421–422.

Ranvier. 1868. Technique Microscopique. Arch. de Phys., **1**, 319–321, 666–670.

Rawitz, B. 1899. Bemerkungen über Karminsäure und Hämatein. Anat. Anzeiger, **15**, 437–444.

Schneider, A. 1880. Über Befruchtung. Zool. Anzeiger, **3**, 252–257.

(Schultz, M.) 1865. Injectionsmassen von Thiersch und Müller. Arch. f. Mikr. Anat., **1**, 148–150.

Schwartz, E. 1867. Über eine Methode doppelter Färbung mikroskopischen Objecte, und ihre Anwendung zur Untersuchung der Musculatur der Darmtraktes der Milz, Lymphdrüsen und anderer Organe. Sitzber Akad. d. Wissensch Wien., Math. Maturw. Classe, **55**, Abt. 1, 671–691.

Schweiger-Seidel, F., and Dogiel, J. 1866. Über die peritoneal Hölle bei Fröschen und ihren Zusammenhang mit dem Lymphgefäss-systeme. Ber. d. K. Sächs Gesel. d. Wiss zu Leipsig, **18**, 247–254.

Spuler, A. 1901. Über eine neue Stückfärbemethode. Deut. Med. Wochnschr., **27**, Vereins-Beilage No. 14, 116.

Van Beneden, E. 1883. Recherches sur la maturation de l'oeuf, et la fecondation. Arch. de Biol., **4**, 265–640.

REACTIONS OF BASIC DYES WITH CYCLIC DERIVATIVES OF AN ACID CHARACTER

WALTER C. HOLMES[1] and RAYMOND M. HANN[2]

(141st Contribution from the Color and Farm Waste Division, Bureau of Chemistry and Soils, U. S. Department of Agriculture, Washington, D. C.)

ABSTRACT.—**The recent discovery that the actual staining agent in the Ziehl-Neelson technic is an addition product of the phenol and the dye employed led the authors to investigate the character of the reaction products of various basic dyes with a considerable variety of cyclic derivatives of a phenolic or acid character. Analytical data are presented which indicate that basic dyes form addition products, in general, with typical phenols. With more definitely acid cyclic derivatives the reaction is primarily metathetical, resulting in the formation of organic salts of the dyes. In some instances both metathesis and addition result. Readers are referred to the following paper for information as to the practical staining value of certain of these compounds.**

In a recent investigation of the mechanism of color retention in the Ziehl-Neelson method for differentiating the tubercle organism in the diagnosis of tuberculosis, carried out by R. W. French of the Army Medical Service, Walter Reed Hospital, Washington, D. C.,[3] it was learned that the phenol does not act as a mordant for the fuchsin, but that the phenol and dye react with the formation of a product which is taken up by the organism. The investigation of the chemical character of this reaction product was undertaken by the senior author.

It was anticipated that the reaction would prove one of simple metathesis, resulting in the formation of the dye phenolate. The phenolate of rosanilin was prepared from the dye base, however, and found to be radically different in properties from the product obtained by treating rosanilin hydrochloride with phenol. It was then determined that the dye (hydrochloride) reacted with dimolecular proportions of phenol and that no elimination of chlorine was involved. The resulting dye-phenol complex was practically identical with the dye in color. It was stable to boiling and could be recrystallized from water or alcohol repeatedly without alteration.

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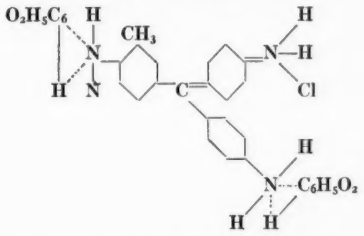
[3]French, R. W. Chemical Structure as Affecting the Staining and Therapeutic properties of Dyes. Stain Techn., **1**, 74. 1926.

On the addition of mineral acids, however, it was decomposed, with the liberation of phenol and the formation of the polyacidic salts of the dye.

From these facts it was apparent that this dye-phenol complex was an addition product. It appears probable that a molecule of phenol is held at each of the "unsaturated" amino groups of the dye by partial valencies or residual affinity and that the complex may be represented by a formula of the following type:



Analogous products were then obtained with phenols and several basic dyes of all important constitutional groups. The complexes color various "acid fast" organisms and spores which resist staining with the dyes themselves and may prove, accordingly, useful biological stains. Certain of them have been found to have decided bacteriocidal and fungicidal potency with but slight toxicity, and are expected to prove valuable bacteriocidal and therapeutic agents.[4]

The present investigation was undertaken to throw further light upon the reactivity of basic dyes with cyclic derivatives of an acid nature.

All of the reaction products of basic dyes and typical phenols which have been investigated have proved addition products. A decided tendency has been observed toward the retention of as many molecules of the phenol as there are unsaturated amino groups in the dye. The reactions of crystal violet and of methylene blue with resorcin are probably typical.

With crystal violet and resorcin the normal reaction product contains two molecules of the phenol in combination with one of the dye (analogous to the rosanilin-phenol complex represented by formula I). This compound is invariably obtained if sufficient resorcin is employed for its formation and its character is not modified by the presence of excess resorcin, however considerable. It is a uniform stable product which may be recrystallized repeatedly without alteration. The unimolecular addition product is formed

[4]Application for patent protection has been made by French and Holmes.

only when insufficient resorcin is used for the formation of the dimolecular product, and then only in limited proportions.

With methylene blue and resorcin the reactions are more complex and the reaction products obtained with excess resorcin are heterogeneous. There are indications of a tendency toward the formation of a unimolecular addition product, as would be anticipated on the assumption of paraquinoid structure in the dye. With increasing excess of resorcin, however, the proportion of resorcin in the product increases progressively and the analytical data indicate that di- and even trimolecular addition products are obtained under extreme conditions. It is also apparent that a limited degree of metathesis also occurs, increasing with increase in the excess of the phenol employed.

The behavior of the phenol may be modified radically by the influence of substituent groups. Thus while phenol yields definite addition products with basic dyes, together with little or no metathetical action, picric acid yields picrates with little or no formation of addition products.

In such reactions between basic dyes and cyclic derivatives containing salt-forming acid substituent groups other than phenolic groups, as have been investigated, metathesis has invariably occurred with the formation of the organic salt of the dye. In some instances no secondary formation of addition products with excess of the acid component has been apparent. In other instances addition product formation has accompanied metathesis but the tendency toward the formation of products of definite molecular proportions has been less evident than in the case of phenols.

The reaction of basic dyes with acid cyclic derivatives may result, accordingly, in the formation of organic salts of the dye or in that of addition products of the normal dye salt, or, finally, in both metathesis and addition. The nature of the reaction in any specific instance is doubtless influenced by the relative basicity and acidity of the reacting substances and the relative solubilities of the possible reaction products.

So far as the authors are aware the formation of addition products between basic dyes and acid cyclic derivatives has not been reported hitherto. Analogous products are familiar, however, altho their character does not appear to have been generally recognized. The oxalate of malachite green and the "zinc chloride double salts" of numerous basic dyes, for example, are obviously addition products of a similar type.

The demonstration of additive structure in basic dye-phenolic complexes is held to afford some measure of support for the assump-

tion of similar structure in the normal dye salts. This assumption has been made by the senior author, on the basis of correlation of color and structure, in order to account for the metachromatic behavior of certain aminated dyes in staining,[5] and for the tautomeric alterations in color and absorption of aminated dyes in general with variation in the concentration of their aqueous solutions.[6] It is of interest that the addition product of basic violet and resorcin undergoes precisely the same tautomeric alteration in aqueous solutions as does the dye itself.

Experimental

The general method of preparation of reaction products employed was that of adding to a concentrated aqueous solution of the dye a concentrated aqueous solution containing twice the amount of the acid component. The reactions occur at room temperatures but hot and nearly saturated solutions were employed, in general, in order to insure maximum yields. In many instances crystalline precipitates were obtained at once, or upon cooling. In other instances tarry deposits were obtained which yielded crystalline products on resolution in hot water or 50% alcohol and subsequent cooling.

In such solubility determinations as were made, the solvent and excess solute were shaken constantly in a constant temperature bath over a period of five days. It was assumed that equilibrium was established by this procedure but this assumption was not checked experimentally.

Rosanilin Phenolate, $C_{20}H_{20}N_3 \cdot OC_6H_5$.

Unimolecular proportions of rosanilin base and phenol were boiled up with alcohol on the steam bath and the solution evaporated to dryness. The product was non-crystalline and only very slightly soluble in water. The remarkable colloidal behavior of its aqueous solution has been reported.[7]

Phenol Rosanilin Hydrochloride, $C_{20}H_{20}N_3Cl \cdot 2C_6H_5OH$.

A moderate excess of phenol was added to an aqueous solution of the dye and the solution distilled with steam until no further phenol came over in the distillate. The solution was evaporated and the residue recrystallized from hot water. $TiCl_3$ consumption (cc. 0.1

[5]Holmes, Walter C. The Chemical Nature of Metachromasy. Stain Techn., **1**, 116. 1926.

[6]Holmes, Walter C. The Influence of Variation in Concentration on the Absorption Spectra of Dye Solutions. Ind. & Eng. Chem., **16**, 35. 1924.

[7]Holmes, Walter C. Colloidal Phenomena in Dye Solutions. J. Am. Chem. Soc., **49**, 790. 1927.

$NTiCl_3$ per gm. of dye) of the vacuum-dried product, 37.42, 37.30 cc. (theoretical, 38.03 cc.). Chlorine found 6.42% (theoretical, 6.72%).

Resorcin New Fuchsin Hydrochloride, $C_{22}H_{24}N_3Cl \cdot 2C_6H_4(OH)_2$.

The $TiCl_3$ consumption of the vacuum-dried recrystallized product was 33.88, 34.02 cc. (theoretical, 34.13 cc.). One liter of a saturated aqueous solution at 26°C. contains 9.4 gm. One liter of a saturated solution in 95% alcohol at 26° contains 57.4 gm.

Hydroquinone New Fuchsin Hydrochloride, $C_{22}H_{24}N_3Cl \cdot 2C_6H_4(OH)_2$.

Nitrogen determinations on the air-dried product gave 6.14, 6.22% (theoretical, 7.07%). After thoro drying the $TiCl_3$ consumption was 33.86, 33.90 c.c. (theoretical, 34.13 c.c.).

Resorcin Crystal Violet Hydrochloride, $C_{25}H_{30}N_3Cl \cdot 2C_6H_4(OH)_2$.

The crude crystalline product contained 6.16, 6.41 and 6.03% N. (corrected for 1.97% moisture = 6.31% N ave.; theoretical, 6.69%). Chlorine found 5.56% (theoretical 5.65%). In a series of precipitations of 20 gm. lots of dye with 5, 10, 20 and 40 gm. of resorcin the nitrogen values of the products obtained were, respectively, 7.10, 6.70, 6.33 and 6.35%. After recrystallization from hot water the material which contained 6.70% N gave values of 6.81 and 6.79%. The absorption of the addition product in water, and the character of its modification with variation in concentration and with acids, is identical with that of the dye itself; see Chart 1.

The addition product is much less soluble in water than the dye. One liter of a saturated aqueous solution at 26°C. contains 2.8 gm. of addition product and 16.8 gm. of crystal violet. One liter of a saturated solution in 95% alcohol at 26°C. contains 138.4 gm. of addition product and 138.7 gm. of dye.

Hydroquinone Crystal Violet Hydrochloride, $C_{25}H_{30}N_3Cl \cdot 2C_6H_4(OH)_2$.

The crude crystalline product contained 6.13, 6.09% N. (theoretical 6.69%). Recrystallized from hot water the $TiCl_3$ consumption was 31.60 cc. (theoretical, 31.85 cc.). One liter of saturated aqueous solution at 26°C. contains 3.0 gm. of addition product. One liter of a saturated solution in 95% alcohol contains 83.9 gm.

Pyrocatechin Crystal Violet Hydrochloride, $C_{25}H_{30}N_3Cl \cdot 2C_6H_4(OH)_2$.

The crude crystalline product contained 6.11, 6.30% N (theoretical 6.69%). Recrystallized from hot water the $TiCl_3$ consumption was 31.68 cc. (theoretical, 31.85 cc.). One liter of saturated aqueous solution at 26°C. contains 7.9 gm. of addition product. One liter of a saturated solution in 95% alcohol contains 249 gm.

Chlorhydroquinone Crystal Violet Hydrochloride, $C_{25}H_{30}N_3Cl \cdot 2C_6H_3Cl(OH)_2$.

The recrystallized product gave N values of 5.90 and 5.89% (theoretical, 6.03%).

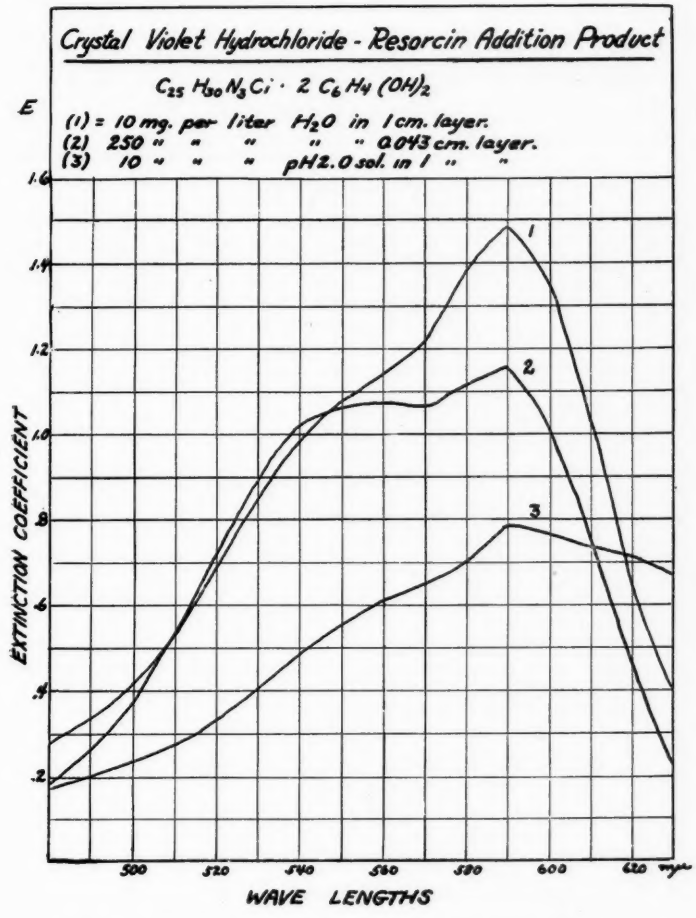


Chart 1. Absorption curves of the resorcin crystal violet hydrochloride addition product.

Pyrogallol Crystal Violet Hydrochloride, $C_{25}H_{30}N_3Cl \cdot 2C_6H_3(OH)_3$.

Nitrogen found, 6.16, 6.22% (theoretical, 6.37%).

Phloroglucin Crystal Violet Hydrochloride, $C_{25}H_{30}N_3Cl \cdot 2(C_6H_3(OH)_3 + 2H_2O)$.

Nitrogen found in the air-dried product, 5.59, 5.52% (theoretical 5.74% N). The affinity of phloroglucin for basic dyes was noted by Waage.[8]

β-Naphthol Crystal Violet.

Nitrogen found in the crude air-dried product, 4.51 and 4.53%. After recrystallization the nitrogen content was 4.76%. The theoretical dimolecular addition product contains 5.23% N.

Crystal Violet Picrate, $C_{25}H_{30}N_3 \cdot OC_6H_2(NO_2)_3$.

The salt is almost completely insoluble in water and might well serve for the gravimetric evaluation of the dye. It was recrystallized from 50% alcohol. Nitrogen found, 13.77, 13.61, 13.89% (theoretical 13.76%). Chlorine was absent. The addition of picric acid to a solution of the addition product of crystal violet and resorcin resulted in the incomplete precipitation of a product of unknown constitution containing only 7.74% nitrogen.

Crystal Violet Acid Phthalate, $C_{25}H_{30}N_3 \cdot O_4C_8H_5$.

The crude product obtained with phthalic acid contained 5.90% N and no chlorine. On recrystallization the nitrogen rose to 7.50, 7.58%. The acid phthalate of the dye contains 7.82% N. The nitrogen content of a second product obtained by the addition of acid potassium phthalate to an aqueous solution of the dye was 7.76, 7.65 and 8.00%.

Crystal Violet 1-Naphthol-4-Sulfonate, $C_{25}H_{30}N_3 \cdot SO_3 \cdot C_{10}H_6 \cdot (OH)$.

Nitrogen found, 6.72, 6.74% (theoretical, 7.06%).

Crystal Violet 1-Naphthol-8-Amino-3:6-Disulfonate, $(C_{25}H_{30}N_3)_2 \cdot (SO_3)_2 C_{10}H_4NH_2$.

Nitrogen found, 8.86% (theoretical, 9.24% N).

The reaction product of crystal violet and aspirin gave nitrogen values of 6.39 and 6.42%.

Resorcin Methylene Blue Hydrochloride.

The original crude crystalline product, obtained with the dye hydrochloride, contained 9.15% N and 6.69% Cl (corrected for 9.2%

[8]Waage, Th. Ueber das Vorkommen und die Rolle des Phloroglusins in der Pflanze. Ber. d. d. Bot. Ges., **8**, 250. 1890.

moisture). A similar product, containing 9.28% N, was obtained from the zinc chloride double salt of the dye. The monomolecular addition product contains 9.78% N and 8.25% Cl. A series of precipitations was carried out in which 5 gm. of the dye hydrochloride in 75 cc. of water was precipitated with 100 cc. of resorcin solution containing 1.25, 2.5, 5, 10, and 25 gm. of the phenol. The nitrogen contents of the unwashed products were, respectively, 8.99, 8.59, 7.80, 7.60 and 6.24%. In a second series 20 gm. of hydrochloride was precipitated with 7, 20 and 100 gm. of resorcin and the precipitates washed. The resulting products contained, respectively, 9.12% N and 7.19% Cl, 8.35% N and 6.51% Cl and 6.63% N and 4.75% Cl.

The reaction product obtained by precipitating the dye hydrochloride by the addition of twice its amount of hydroquinone gave nitrogen values of 9.26 and 9.11%. With pyrocatechin under similar conditions the product gave nitrogen percentages of 7.67 and 7.63%. The product obtained with new methylene blue (NN) and hydroquinone gave nitrogen values of 8.40 and 8.41%.

Methylene Blue Salicylate, $C_{16}H_{18}N_3.CO_2.C_6H_4.OH+2H_2O$.

The crude product obtained with the dye and sodium salicylate was recrystallized from water and alcohol. The nitrogen values obtained were, respectively, 8.47 (8.42 and 8.52) % and 8.67 (8.72 and 8.62) %. The combined products were recrystallized from 90% alcohol and gave 8.97 (8.88 and 9.06) % N. After another recrystallization it gave 8.83% N.

A similar product was obtained by the addition of salicylic acid to the dye which contained 8.92 (8.90 and 8.93) % N. It may be noted, however, that the normal salicylate is not invariably formed from the acid and dye. In one test, the conditions of which were not recorded, a product was obtained which crystallized from alcohol with only 6.32 (6.42 and 6.21) % N.

Methylene Blue Acetyl Salicylate, $C_{16}H_{15}N_3S.CO_2.C_6H_4.O_2C_2H_3$.

Nitrogen found, 8.91, 9.17% (theoretical, 9.07%).

Tetraethyl Thionin Acetyl Salicylate, $C_{20}H_{26}N_3S.CO_2.C_6H_4.O_2C_2H_3$.

$TiCl_3$ consumption, 38.32, 38.25 cc. (theoretical, 38.50 cc.).

Azure C[9] *Acetyl Salicylate*. $C_{13}H_{12}N_3S.CO_2.C_6H_4.O_2C_2H_3$.

$TiCl_3$ consumption, 46.88, 47.08 cc. (theoretical, 47.47 cc.).

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[9]Holmes, W. C., and French, R. W. The Oxidation Products of Methylene Blue. Stain Techn., **1**, 17. 1926.

Methylene Blue O-Toluate, $C_{16}H_{18}N_3S.CO_2.C_7H_7$.

Nitrogen found, 9.84% (theoretical, 10.00%).

With phthalic acid methylene blue gave a product which contained 7.54 (7.58, 7.50) % N.

Application

A study of the value of some of these products in staining procedures has been made by R. W. French of the Army Medical Museum and is discussed in the following paper.

NOTES ON STAIN APPLICATION

R. W. French, *Army Medical Museum, Washington, D. C.*

Preliminary studies have been made of some of the new dye compounds described in the preceding paper.[1] The results have apparently shown that they may have some practical application in staining procedures. In general all act similarly to the customary phenol mixtures employed—for instance an aqueous solution of new fuchsin hydroquinone was found to stain the tubercle bacilli very well and similarly to the usually employed Ziehl-Nielson carbol-fuchsin. The resorcinolate and phenolate preparations also acted in a similar manner. In general the compounds, when sufficiently soluble, appear to afford very intense stains, which, however, are difficult to differentiate. In fact, to date, this has precluded their practical application in tissue studies.

In smears of syphilitic material from a primary lesion crystal violet resorcinolate stained *Treponema pallidum*, satisfactorily and rapidly; but it also stained detritus, et cetera, so intensely that the organism was not especially clear against the resultant background.

Before these compounds may be employed to good advantage in tissue study, means for satisfactory differentiation is necessary. Several of the crystal violet compounds have given evidence of possible interest in the study of elastic tissue degeneration, tho so far results have not been sufficiently consistent to allow any definite conclusions, as regards possible employment in the study of pathological conditions.

[1]Holmes, W. C., and Hann, R. M. Stain Techn., 3, 122. 1928.

INVESTIGATION OF THIAZIN DYES AS BIOLOGICAL STAINS

II. INFLUENCE OF BUFFERED SOLUTIONS ON STAINING PROPERTIES

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ABSTRACT.—**The effect of buffer solutions of varying reaction upon staining fixed sections with thionin, azures A, B, and C, and methylene blue has been studied. The buffer solutions were employed in one of three different ways: for pre-treatment of the sections, for post-treatment, or as solvents for the dyes. Regardless of the method of employing the buffer solutions it was found that the intensity of staining increased with increasing pH-values (a fact which is generally known to be true in the case of basic dyes). It is not certain whether this effect is due to varying the H-ion concentration or to altering the salt content of the solution, or to both. It was also noticed that there was one point where the staining intensity increased most rapidly. This point was either between pH 5 and pH 6 or between pH 6 and pH 7, its position varying with the method of fixation and of applying the buffer solutions. It was further observed that between pH 5 and pH 7 there were always more pronounced metachromatic effects than with either more acid or more alkaline buffer solutions.**

In the earlier paper of this series[1] it was shown that in the thiazin series of dyes there is a relation between the extent of ethylation or methylation of the dye and its staining properties. In the course of this investigation it became evident that the staining action of any given dye was greatly influenced by other factors, such as reaction of the material stained, composition and reaction of the staining solution, and so forth. To secure consistent results it was necessary to control all these factors very carefully.

At the end of the paper just referred to it was stated that one of the points calling for further investigation was "the effect of buffer solutions of different reactions upon these dyes, not only when incorporated in the staining solution, but also when used for pretreating or post-treating the slides." The present work was carried on to study this point.

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[1]Haynes, R. Investigations of thiazin dyes as biological stains. I. The staining properties of thionin and its derivatives as compared with their chemical formulae. Stain Techn., **2**, 8–16. 1927.

It must be realized, however, that in employing buffer solutions, the H-ion concentration is not the only factor that is varied. Buffer solutions are mixtures of salts, and it is well known that staining with anilin dyes is profoundly influenced by salts as well as by acids and bases. If one accepts the theory that dyeing is a process of polar adsorption, the effect of salts and of reaction may be considered the same with only a difference in degree; for practical purposes, however, one must distinguish between the two effects.

In textile dyeing this has long been recognized and has been pointed out by many workers in histological staining. Wertheimer[2] has shown that with living membranes "anions increase the staining of frogs' skin with basic dyes with increasing valence, cations behave in the opposite manner. With acid dyes the reverse is true. The effect which a salt has on staining is also an expression of its constituent anions and cations." The effect of acids and bases on histological staining has perhaps received more general attention by histologists, undoubtedly on account of the significance that has been attached to the iso-electric point of proteins.

Naylor[3] has pointed out the importance of H-ion concentration in staining sections of plant tissue; but inasmuch as he used a strong base and a weak acid in making up his solution varying in pH-value, he probably also had some salt effect in addition to that of H-ion concentration.

Mommsen[4] in studying the iso-electric point of red blood cells has used buffered staining solutions. Stearn and Stearn[5] have studied the effect of H-ion concentration in staining bacteria, making use of buffer solutions in determining the iso-electric point of the tissues rather than in working out practical staining technics.

To show the salt effect alone on the staining of tissues, it is necessary to use neutral salt solutions of varying concentrations; whereas, to show the effect of reaction of the staining solution free from the salt effect, it is necessary to use strong acids and bases in varying concentrations.

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[2]Wertheimer, E. Über den Einfluss von Ionen auf Farbstoffe und die Anfärbbarkeit von Gewebe. Pflüg. Arch. Ges. Physiol., **202**, 383–394. 1924.

[3]Naylor, E. E. Hydrogen-ion concentration and staining of sections of plant tissue. Amer. J. Bot., **13**, 265–275. 1926.

[4]Mommsen, H. Über die elektrostatische Ladung von Zellen des menschlichen Blutes, ein Beitrag zur Frage der Azido- und Basophilie. Folia Hemat., **34**, 50–64. 1927.

[5]Stearn, E. W., and Stearn, A. E. Chemical mechanism of bacterial behavior. I. Behavior toward dyes. Factors controlling the Gram stain. J. Bact., **9**, 463–477. 1924.

In actual practice it is difficult to control the reaction of a staining solution without altering its salt content, because of the lack of buffer effect in solutions containing only strong acids and bases; and preparations made with them are, moreover, likely to fade. For such reasons the most convenient method of altering the staining intensity is by means of buffer solutions—whereby both the H-ion concentration and the salt content are varied simultaneously.

In the present investigation the effect of a series of buffer solutions on the staining properties of thionin and certain of its homologs has been studied. The fact has been kept in mind, however, that two factors capable of influencing staining results are being varied.

Materials and Methods

The material stained was fixed tissue embedded in paraffin and sectioned six microns thick. Tissue from cat, rat, and mouse, including brain, intestine, bone, spleen, and kidney, was used with one or more of the following fixing solutions in each instance: Zenker, Helly, Bouin, Ohlmacher, alcohol, and picrosulfuric acid. The dyes employed were: tetra-ethyl thionin, methylene blue, azure B, azure A, azure C, and thionin.[6]

Use was made of Kolthoff's[7] buffer solutions of succinic acid, potassium acid phosphate, and borax, selecting those representing the following pH-values: 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0. The reaction was checked up by means of indicators before adding the dye and was found to be approximately correct. The exact reaction of the dye solutions themselves was not determined; but it is believed that the addition of weakly acid salts (such as the dyes in question) to strongly buffered solutions would not alter the reaction enough to effect the results of the present investigation.

The slides were treated in three different ways: Series 1, treated with a 1% solution of the dye in the buffer solutions (in this series thionin was partially precipitated in all solutions more alkaline than pH 5); Series 2, pre-treated with the buffer solutions, then stained, dehydrated, and mounted; Series 3, stained, then treated with the buffer solutions, dehydrated, and mounted.

Results

General. As would be expected with basic dyes, a gradual increase in intensity of staining occurs from pH 3 to pH 9, with all the dyes

[6]These dyes are listed in order of decreasing alkylation. For exact formulae see the preceding paper. Stain Techn., **2**, 9. 1927.

[7]Kolthoff, I. M. A new set of buffer mixtures that can be prepared without the use of standardized acid or base. J. Biol. Chem., **63**, 135–141. 1925.

used, irrespective of the material, the fixing fluid, or the method of employing the buffered solution. All the figures illustrate this tendency.

Series 1. Fig. 1 is a reproduction of a series of photographic prints made by placing the slides directly on the sensitized paper, exposing and developing; results are therefore in negative, the most deeply stained sections appearing lightest. In this figure there is given a comparison of the five dyes made up in buffer solutions as

Fig. 1. Series 1. Direct impressions on photographic paper of sections of two-day rat kidney, fixed in Helly's fluid. Stained 30 minutes in a 1% solution of the dyes in Kolthoff's buffer series.

illustrated by their action on sections of rat kidney fixed in Helly's fluid. These prints show plainly the tendency for the depth of staining to increase with decrease in H-ion concentration; in some instances there is a slight discrepancy due to irregularities in thickness of the sections, but in general the figure gives an accurate picture of the intensity of staining. It will be noticed that depth of staining with methylene blue and azure B is less affected by the change in reaction than with azure A and azure C. The results with thionin

are not comparable with the others because of the precipitation of the dye in the four most alkaline solutions.

In one respect Fig. 1 does not tell the whole story concerning these slides: altho the sections stained with any one dye were all exposed simultaneously, the sections stained with the different dyes were exposed at different times and the length of exposure slightly varied. Hence a comparison up and down can always be made in this figure; but not right and left. This figure does not illustrate, for example, the relative intensities of the various sections stained in solutions of

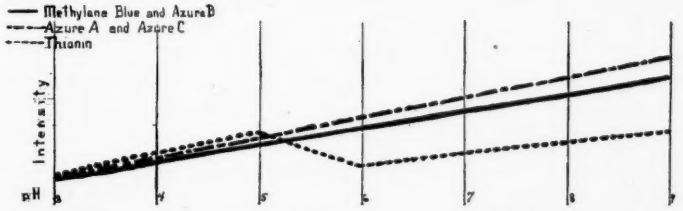

Fig. 2. Series 1. Estimated depth of staining plotted against pH-value. Sections of rat kidney (fixed in Helly's fluid) stained in 1% solutions of the dyes in Kolthoff's buffer series (same material as in Fig. 1).

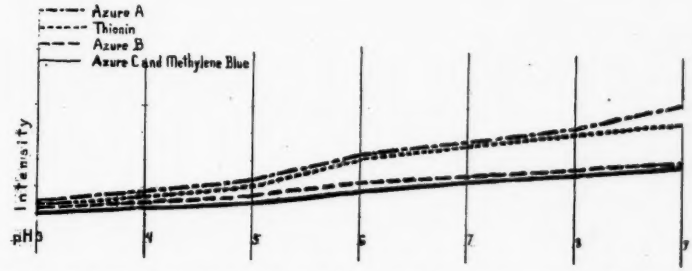

Fig. 3. Series 2. Estimated depth of staining plotted against pH-value. Sections of rat kidney (fixed in Helly's fluid) pre-treated 12 hours in Kolthoff's buffer solutions, then stained 30 minutes in 1% solutions of the dyes.

pH 7. To illustrate this point, the graphs given in Fig. 2 and Fig. 3 have been drawn. In these graphs the intensities are only estimated and hence the actual position of each curve is not scientifially accurate.

In interpreting these results it becomes of interest to recall the formulae of the five dyes, as discussed in the preceding paper. Methylene blue, it will be remembered, is tetramethyl thionin, while azure B has three methyl groups, azure A two and azure C only one. Thus, with the exception of thionin, the intensity of the staining at

pH 9 is in inverse proportion to the extent of methylation of the dye; or in other words, increasing the basisity of the dye bath has the more effect on intensity of staining the fewer the number of methyl groups in the molecule.

The fact that thionin is out of place in this series is readily explained by its partial precipitation in the more alkaline solutions, as a result of which the staining was done with a very dilute solution of the dye. The next series it was hoped would give a fairer comparison between this dye and the others.

Series 2. Examination of slides pre-treated with buffer and then stained with 1% solution of the dye (see Fig. 3) showed the following results: With each dye an increase in staining occurred, as in the preceding series, from pH 3 to pH 9. A comparison of intensity of staining action of the five dyes at each pH-value is shown in Fig. 3 for a typical series fixed in Helly's fluid. It will be noted that azure C

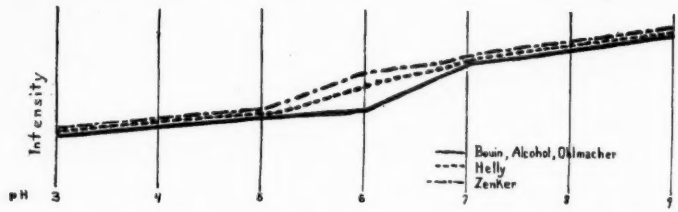

Fig. 4. Series 2. Comparison of results following the different fixing solutions. Estimated depth of staining plotted against pH-value. Based upon sections stained with azure A after treating 12 hours with Kolthoff's buffer solutions; but results apply in general to the other dyes also.

was less intense than azure B. Inasmuch as azure C was as intense as azure A in buffer solution, its faint staining in this test is probably due to residual acid or salt impurities in the dye sample. It will be noticed that in series 2, thionin is almost equal to azure A in intensity of staining. Here the stain did not precipitate; if the dyes had been used in dilute enough solutions in the buffers so that thionin would not precipitate, it would undoubtedly have been as intense as azure A in series 1 also.

Thus series 2 resulted, just as anticipated, in placing thionin in essentially the position in the series where its formula would lead one to expect it to be. The only dye out of order in this series was azure C; but it is felt that little stress can be laid on any such results, for the use of unbuffered staining fluids allowed any impurity that might be present in the dye itself to have a great influence on the staining.

Another point observed in this series of slides is illustrated in Fig. 4. With tissue fixed in Zenker's fluid, intensity of staining increased much more between pH 5 and pH 6 than between any other two points in the pH-scale investigated. In the case of tissue fixed in alcohol, Bouin's or Ohlmacher's solutions, a similar abrupt change occurred between pH 6 and pH 7, but no marked increase from pH 5 to pH 6. With Helly's fixative the increase was much more uniform, altho greater between pH 5 and pH 6 than between pH 6 and pH 7. In this connection it is well to recall that Helly's fluid is a modification of Zenker's, in which acetic acid is replaced with formalin.

Paralleling this sudden increase in intensity of staining, a change in color of the sections was observed. At the same point where the intensity increased abruptly there was a distinct change from the purplish shade at the acid end of the series to a greenish or bluish

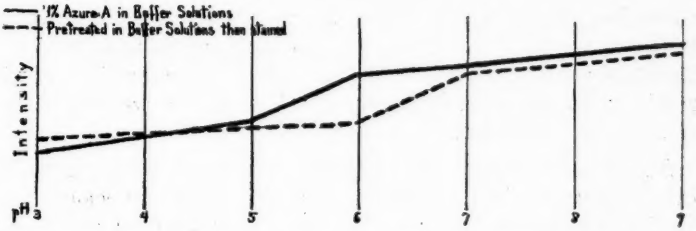

Fig. 5. Comparison of series 1 with series 2. Estimated depth of staining plotted against pH-value. Based upon sections of crow-brain, fixed in alcohol, and stained with azure A; but results apply in general to the other dyes as well (except thionin in series 1).

shade which characterized the alkaline end. This change in color was due to the fact that under the alkaline conditions certain tissue elements assumed a green color, as illustrated by the non-medulated nerve fibers in crow-brain (alcohol fixed) and smooth muscle fibers in the tunica muscularis of intestine (Bouin fixation). It will be understood, of course, that the exact nature of the color change which occurs at this point is governed by the metachromatic nature of the dye, and is quite different with thionin from what it is with methylene blue. This is discussed below under the heading "metachromatic effects."

If this abrupt change in color and intensity occurred in every instance between pH 5 and pH 6 one would be tempted to draw the conclusion that it depended not upon reaction but upon the nature of the salts present in the buffer solutions; for between these two

points in the Kolthoff series, one changes from borax-succinic-acid to borax-phosphate mixtures. Inasmuch, however, as the greatest change occurred at this point only with Zenker and Helly fixation, while with material fixed in the other fluids it occurred between two buffer solutions both containing phosphate, the natural conclusion is that the effect cannot be due to the change in the nature of the salts used.

Fig. 5 shows a comparison between series 1 and series 2 in this same respect, as illustrated by sections fixed in alcohol and stained with azure A. It will be seen that with this method of fixation the abrupt change in staining intensity occurred between pH 5 and pH 6 in series 1 (where the stains were dissolved in the buffer solutions) instead of between pH 6 and pH 7 as in series 2 (where the buffer solutions were used for pre-treatment).

Series 3. No chart is given to indicate the results of this last series in which the buffer solutions were used for post-treatment of the stained slides. The general results were the same as in series 2, in that the order of the intensity of the various dyes was identical. The slides were all more faintly stained, however, because the method requires washing for some time after staining, a process which removes considerable of the dye.

Metachromatic Effects. In all of the series, metachromasy is most evident between pH 5 and pH 7; below pH 5 the green color in the red blood cells, cilia, cell walls, and fibers is very faint or absent, while at pH 8 the staining is so intense that the green and purple shades are not distinguishable.

It may be recalled from the earlier paper, that comparative metachromatic staining with these dyes in buffer solutions of a given pH-value is as follows: Tetra-ethyl thionin shows no metachromasy, everything staining greenish blue, the other five dyes (methylene blue, azure B, azure A, azure C, and thionin) are metachromatic. Staining effects suggest the presence of at least three dyes instead of one: green, blue and red. The red increases and the green decreases with a decreasing number of alkyl groups. Thus, altho there are certain structures which appear green even when stained with thionin, and others that are reddish with methylene blue, the most intensely green portions of a methylene blue slide are not so bluish as those of a thionin slide; similarly the most pronouncedly red portions of a thionin slide are less blue than those of a methylene blue slide.

This comparison holds true only if slides stained at any given reaction are taken into account. There was, however, as indicated

above, a decided difference in the amount of metachromasy produced by any one dye with varying reaction of the staining fluid. This relation held true in general no matter which dye was considered; but each dye has its peculiarities in this regard and the exact effect of any particular buffer solution upon any one of these dyes is not yet entirely understood. Nor is it certain whether the effect is due to varying the H-ion concentration or to altering the salt content of the solution or to both. Such points must be left for future study.

Conclusions

Any desired depth of staining may be obtained with the dyes of this series merely by dissolving the dye in the proper buffer solution. Pre-treatment and post-treatment of the slides give similar results, but the staining is likely to be fainter, especially in the case of those slides which are post-treated with buffer. The best H-ion concentration for securing a metachromic preparation is in the pH-range between 5 and 8, as some of the elements do not stain below 5, and above 8 staining is too intense to differentiate the various colors.

WRIGHT'S AS A DIFFERENTIAL SPORE STAIN

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ABSTRACT.—**A method of differential spore staining utilizing Wright's stain diluted one to five in a phosphate buffer solution of pH 7.6 and following the general technic of the Dorner method is outlined. Spores are stained a deep blue while the cytoplasm of the sporangium is stained a pinkish red.**

Upon examining smears of fecal material stained with hot, diluted Wright's stain in the course of other experimental work, certain bacteria with red cytoplasm and what appeared to be a very faint pale blue spore were seen. It occurred to me that if these were actually bacterial spores Wright's stain might be utilized as a differential spore stain.

A pure culture of a spore-forming organism, isolated from the air but not identified, was smeared and stained with Wright's in a number of ways—both hot and cold. The results were no more than suggestive, however, as the spore body either failed to stain, or was stained so faintly as to be seen with difficulty.

It was thought that, if a more intense heat could be applied without evaporating the methyl alcohol of the staining solution, the spore body would be stained a deeper blue as a result. Accordingly an attempt was made to adapt the technic of the Dorner method of spore staining as outlined in the "Manual of Methods for Pure Culture Study."[1] After experimenting with various procedures and dilutions of the stain the following method was found satisfactory:

A heavy suspension of the organism to be stained is made in 0.4 cc. of distilled water in a fairly large test tube (150x13 mm.). To this suspension 0.1 cc. of Wright's staining solution (50 to 100 mg. to 60 cc. of methyl alcohol) is added. The tube is then tightly stoppered to prevent evaporation of the alcohol and immersed in boiling water for about ten minutes. It is removed from the water bath and allowed to cool for about half a minute to prevent the contents from bubbling out when it is opened. Loopfuls of the stained organism are then spread on slides and dried. Examination shows the cytoplasm of the sporangium stained a reddish brown and the spore bodies a fairly intense blue.

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[1]Committee on Bacteriological Technic. Manual of Methods for Pure Culture Study of Bacteria. Published by Society of American Bacteriologists, Geneva, N. Y. A loose-leaf publication. 1923; with revisions to date.

If desired, a loopful of the organism may be mixed with an equal quantity of saturated aqueous solution of nigrosin, as in the Dorner[2] technic, and then spread on slides. The spores are then more plainly seen. Nigrosin weaker than saturated solution may be used with the Wright's stain, for it is a very powerful decolorizing agent for the red in the cytoplasm. As high a dilution as one to 100 renders the bacterial cell, except the spore, completely colorless.

As the contrast between the spore and the remainder of the cell was not very striking, it was sought to still further intensify the blue. It seemed plausible that this might be accomplished by controlling the hydrogen-ion concentration of the staining mixture. Phosphate buffer solutions ranging in pH from 6.0 to 8.0 in steps of 0.2 were made up. These were used in place of the distilled water for making the bacterial suspension. A reaction of pH 7.6 gave the optimum results.

The buffer of pH 7.6 may be prepared by mixing 89 cc. of tenth molecular solution of Na_2HPO_4 (14.21 gms. sodium phosphate, dibasic, anhydrous, Merck's reagent, per liter) with 11 cc. tenth molecular solution of KH_2PO_4 (13.62 gms. potassium phosphate, monobasic, anhydrous, Merck's reagent, per liter). The spores stained at this pH were almost black, while the brownish red of the cytoplasm was slightly paler, thus greatly increasing the contrast between spore body and cytoplasm. The preparations thus made are much better than those prepared by the usual counter-staining methods.

It is interesting to note that pH-values above 7.0 cause the sporangium to be permeable to the nigrosin, thus rendering cells stained at this reaction unfit for spreading with it as described above.

Some of the preparations when viewed under the microscope are seen to be littered with a fine reddish precipitate which obscures the picture. Such slides can be saved by flooding them for a second or two, as rapidly as possible, with methyl alcohol, or preferably, undiluted Wright's stain, and washing with tap water.

The spores stained by this method are distinctly smaller than those stained by the Dorner method, as well as by all other methods with which I am familiar. This suggests that the collected chromatin of the spore is stained while the spore wall itself is left unstained. This interpretation is further supported by the fact that free spores when spread with nigrosin almost always show a narrow colorless zone surrounding them which is not seen with the Dorner method where the black of the nigrosin comes directly to the red of the spore body.

[2]Dorner, W. Ein neues Verfahren für isolierte Sporenfarbung. Jahrb. d. Schweiz., **36**, 595–7. 1922.

Altho it cannot be claimed that the method offers a more striking demonstration of bacterial spores than the Dorner method, it seems to give better preparations than the older counterstaining methods. If upon further study it should be found that only the material contained within the spore wall is stained, the method will then offer a means of studying more completely another morphological detail of bacteria.

NOTES ON TECHNIC

TYPES OF SAFRANIN AND THEIR USE.—The safranin samples on the market vary in regard to the percent of actual dye present, and—what is perhaps more important—in the nature of the salts or other ingredients making up the remaining bulk of the sample. There is apparently little correlation between the staining properties of the safranin samples and their dye content. Of the samples sent in for certification in the last five years, some which were judged poor had a dye content above 80%, some which were judged fair had a dye content above 90%, while two which were judged good were 54.9% and 76.2%. The dye itself was exactly the same in all these samples, so far as could be judged by spectrophotometric analysis.

It is thus apparent that variations in the staining properties of the various safranin samples can not be explained by dye content or by the nature of the dye itself. It is probable, therefore, that the salt content is to some extent responsible for the staining properties.

One more thing must be considered in the judgment of a given safranin sample—namely, the staining technic in which it is employed. In the staining of plant chromosomes, for instance, some investigators prefer a technic in which the slide remains in a solution of safranin for twelve hours or more. These workers prefer a safranin rather weak in staining action; for samples which stain strongly give a very muddy appearance to the cytoplasm upon differentiation after staining for this length of time, and thus the chromatin of the division figures does not stand out brightly against a clear background. On the other hand, some investigators prefer a rapid staining technic; and for them samples of safranin with intense staining action are the only satisfactory ones. With these samples, if the slides are stained for five minutes and differentiated rapidly, the chromosomes will appear a bright red and the background will be clear. Obviously the more weakly staining safranins are not usable in this type of technic, for a sufficiently intense stain is not obtained in such a short time. This conclusion is drawn partly from a comparison of reports on safranin sent in by members of the Stain Commission, and partly by the writer's own work with aqueous solutions of the dye.

At the present time both these types of safranin are on the market under Commission certification. Inasmuch as each type has found special favor in the hands of certain workers the Stain Commission has never felt justified in rejecting a sample because others, employing it in a different technic, have found it unsatisfactory. There is no way to tell these two types apart from the label; and the purchaser

can learn which type he has only by seeing which technic gives best results with his sample.

Attention is called to this difference between safranin samples to help cytologists explain their poor results in case they have found some market sample unsatisfactory in their technic. It is recommended that such dissatisfied users either vary their technic or write to the Commission laboratory for suggestions as to where to obtain a safranin better adapted to their technic.—*Rachel Haynes, Geneva, N. Y.*

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CERTIFIED STAINS

The following stains have now been put on a certification basis by the Commission on Standardization of Biological Stains:

Anilin blue, water soluble	Martius yellow
Bismarck brown Y	Methyl green
Brilliant cresyl blue	Methyl orange
Brilliant green	Methyl violet
Carmin	Methylene azure
Congo red	Methylene blue
Cresyl violet	Methylene violet
Crystal violet	Neutral red
Eosin, alcohol soluble	Nile blue A
Eosin, bluish	Nigrosin
Eosin, yellowish	Orange G
Ethyl eosin	Orange II
Fuchsin, acid	Phloxine
Fuchsin, basic	Pyronin
Gentian violet	Rose Bengal
Haematoxylin	Safranin O
Indigo carmin	Sudan III
Janus green B	Sudan IV
Jenner's stain	Tetrachrome stain (MacNeal)
Light green, S. F., yellowish	Thionin
Malachite green	Toluidine blue

Wright's stain

Certification on one or more of these stains has been issued to each of the following manufacturers and standardizers of biological stains: Coleman and Bell, Empire Biochemical Co., Hartman-Leddon Co., McAndrews and Forbes, National Anilin and Chemical Co., Providence Chemical Laboratories. A stain may be ordered from one of these companies direct when it is known which companies have submitted to the Commission the particular stain desired: or it may be secured thru one of the dealers in general laboratory supplies: In the latter case the purchaser must be sure to specify Commission certified stains. In doing so he will be sure that he is purchasing from a batch of which a sample has been tested by one or more experts skilled in its use.

The Commission has now on sale a short book (150 pages) entitled Biological Stains, written by the chairman of the Commission with the collaboration of the other members of its executive committee. The subject is treated primarily from the viewpoint of the biological laboratory worker. The chemistry of dyes is discussed, but with the idea of giving information desired by the biologist rather than by the chemist. The uses of the stains are discussed, together with a brief account of the theories of staining.

The price of the book is $2.50. Orders may be placed with the chairman of the Commission: H. J. Conn, Lock Box 299, Geneva, N. Y.